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(54) Title: ERA COMPLEXES AND USES THEREOF

(57) Abstract

Provided are Era/RNA complexes comprising at least one Era protein and at least one RNA species selected from 16S rRNA, 23S rRNA, 5S rRNA, or mRNA of Era, or an oligomeric fragment, probe, or primer derived from such 16S rRNA, 23S rRNA, 5S rRNA, or mRNA of Era. The formation of such complexes has been found to be necessary for high levels of GTPase activity of the Era protein, and viability of microorganisms in which such complexes are present. Also provided are methods for preparing Era/RNA complexes *in vivo* and *in vitro*, for identifying inhibitors of Era/RNA complex formation and complex stability, for identifying compounds that bind to Era proteins and Era/RNA complexes, and for identifying inhibitors of Era protein enzymatic and ligand binding activities in isolated Era proteins and Era/RNA complexes.

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ERA COMPLEXES AND USES THEREOF

This invention claims the benefit of priority of U.S. Provisional Applications Serial Nos. 60/110,912, filed December 4, 1998; 60/148,362, filed August 11, 1999; and 60/148,914, filed August 13, 1999. The contents of each of these applications is incorporated herein by reference in their entirety.

Background of the Invention

Field of the Invention

The present invention relates to the field of human medicine, particularly the discovery of new antibacterial agents.

Description of Related Art

Era protein is an essential membrane-associated GTPase that is present in microorganisms such as bacteria and Mycoplasma. Era is an essential GTPase that binds GTP and GDP, and hydrolyzes GTP to GDP (Ahnn, J., *et al.*, *Proc. Natl. Acad. Sci. USA*, 83, 8849-8853 (1986); Britton, R. A., *et al.*, *J. Bacteriol.*, 179, 4575-4582 (1997); Britton, R. A., *et al.*, *Mol. Microbiol.*, 27, 739-750 (1998); Chen, S.-M., *et al.*, *J. Biol. Chem.* 265, 2888-2895 (1990); Gollop, N., & March, P. E., *Res. Microbiol.* 142, 301-307 (1991); Gollop, N., & March, P. E., *J. Bacteriol.* 173, 2265-2270 (1991); Inada, T., *et al.*, *J. Bacteriol.* 171, 5017-5024 (1989);
Kawabata, S., *et al.*, *FEMS Microbiol. Let.* 156, 211-216 (1997); March, P. E., *et al.*, *Oncogene* 2, 539-544 (1988); Sato *et al.*, *FEMS Microbiol. Letter 159*, 241-245 (1998); Takiff, H. E., *et al.*, *J. Bacteriol.* 171, 2581-2590 (1989); Wu, J., *et al.*, *Infect. Immun.* 63, 2516-2521 (1995); Zhao, G., *et al.*, *Microbiology* 145, 791-800 (1999)). The *era* gene was first identified in *Escherichia coli*, and was thought to encode a member of the RAS GTPase superfamily based on its sequence similarity to the GTP/GDP binding motifs of RAS (Ahnn, J., *et al.*, *Proc. Natl. Acad. Sci. USA*, 83, 8849-8853 (1986)). Homologues of Era have now been identified in all bacterial genomes sequenced to date

(Fleischmann, R. D., *et al.*, Science 269, 496-512 (1995); Fraser, M., *et al.*, Science 270, 397-403 (1996); Kawabata, S., *et al.*, FEMS Microbiol. Let. 156, 211-216 (1997); Yamashita, Y., *et al.*, J. Bacteriol. 175, 6220-6228 (1993); Zhao, G., *et al.*, Microbiology 145, 791-800 (1999); Zuber, M., *et al.*, Mol. Microbiol. 14, 291-300 (1990); Zuber, M., *et al.*, Gene 189, 31-34 (1997)). Era appears to be highly conserved functionally since *era* genes cloned from diverse bacterial species are able to complement *E. coli* mutants deficient in Era expression (Pillutla, C. R., *et al.*, J. Bacteriol. 177, 2194-2196 (1995); Zhao, G., *et al.*, Microbiology 145, 791-800 (1999); Zuber, M., *et al.*, Mol. Microbiol. 14, 291-300 (1990); Zuber, M., *et al.*, Gene 189, 31-34 (1997)). Percent identities across the microorganisms range from 28-100%. Recently, homologues of Era have also been identified in humans and plants (Britton, R. A., *et al.*, Mol. Microbiol., 27, 739-750 (1998); Ingram *et al.*, 1998). The human homologue of Era is distinct from ras (Britton, R. A., *et al.*, Mol. Microbiol., 27, 739-750 (1998)). Thus, Era does not appear to be a member of the RAS family (Britton, R. A., *et al.*, Mol. Microbiol., 27, 739-750 (1998)).

Several lines of evidence suggest that Era is involved in cell cycle regulation and ribosome assembly, and that the GTP-binding and hydrolysis activities of Era are essential for its biological function (Britton, R. A., *et al.*, J. Bacteriol., 179, 4575-4582 (1997); Britton, R. A., *et al.*, Mol. Microbiol., 27, 739-750 (1998); Gollop, N., & March, P. E., J. Bacteriol. 173, 2265-2270 (1991); Lerner, C. G., *et al.*, FEMS Microbiol. Lett. 95, 137-142 (1992); Lerner, C. G., *et al.*, FEMS Microbiol. Lett. 126, 291-298 (1995); Nashimoto, H. Escherichia coli. In The Translational Apparatus, 185-195 (1993), edited by Nierhaus, H. K., *et al.*, Plenum Press, New York; Nashimoto, H., *et al.*, Mol. Gen. Genet. 199, 381-387 (1985); Nashimoto, H., & Uchida, H., Mol. Gen. Genet. 201, 25-29 (1985)). Mutations affecting the cellular level of Era or the function of Era led to a severe alteration in ribosome assembly, a drastic change in cell morphology, a significant reduction in cell viability, and an apparent suppression of temperature-sensitive mutations in a number of the genes that are involved in DNA replication and chromosome partitioning (Britton, R. A., *et al.*, J. Bacteriol., 179, 4575-4582 (1997); Britton, R. A., *et al.*, Mol. Microbiol., 27, 739-750 (1998); Gollop, N., & March, P. E., J. Bacteriol. 173, 2265-2270 (1991); Nashimoto, H. Escherichia coli. In The Translational Apparatus, 185-195 (1993), edited by Nierhaus, H. K., *et al.*, Plenum Press, New York;

Nashimoto, H., *et al.*, Mol. Gen. Genet. 199, 381-387 (1985); Nashimoto, H., & Uchida, H., Mol. Gen. Genet. 201, 25-29 (1985)). Studies also suggested that Era might be involved in the regulation of energy metabolism since the decrease in the cellular amount of Era altered the ability of *E. coli* to utilize carbohydrates in a minimal medium (Lerner, C. G., & Inouye, M., Mol. Microbiol. 5, 951-957 (1991); Shimamoto, T., & Inouye, M., FEMS Microbiol. Lett. 136, 57-62 (1996)). Together, these results suggest that Era is a multifunctional protein that is involved in the regulation of the cell cycle, protein synthesis, energy metabolism, and DNA synthesis. The mechanisms by which Era regulates these cellular processes remain to be determined, however.

Era consists of two distinct domains (Chen, X. , *et al.*, Proc. Natl. Acad. Sci. USA. 96, 8396-8401 (1999); Zuber, M., *et al.*, Gene 189, 31-34 (1997)). The N-terminal domain of the protein contains the three GTP/GDP-binding motifs that are shared by many different families of GTPases (March, P. E. Mol. Microbiol. 6, 1253-1257 (1992), Pillutla, C. R., *et al.*, J. Bacteriol. 177, 2194-2196 (1995); Zuber, M., *et al.*, Gene 189, 31-34 (1997)). In contrast, the C-terminal domain of Era is highly conserved only among Era proteins, and is thereby unique to the Era family (March, P. E. Mol. Microbiol. 6, 1253-1257 (1992), Pillutla, C. R., *et al.*, J. Bacteriol. 177, 2194-2196 (1995); Zuber, M., *et al.*, Gene 189, 31-34 (1997)). Consistent with this view, studies have indicated that the C-terminal domain of the *Streptococcus pneumoniae* Era protein was responsible for the attachment of the Era protein to the cytoplasmic membrane (Zhao, G., *et al.*, Microbiology 145, 791-800 (1999)). This membrane-binding activity of Era appears to be essential for its biological function in the cell (Zhao, G., *et al.*, Microbiology 145, 791-800 (1999)). Thus, the C-terminal domain of Era, although its function is unknown, most likely plays a direct role in the regulation of the cell cycle and protein synthesis.

The *era* gene is a "minimal gene set" counterpart. The minimal gene set proteins are thought to be essential for viability, and are useful targets for the development of new antibacterial compounds. Thus, cells that carry knockout mutations in *era* are nonviable.

Widespread antibiotic resistance in common pathogenic bacterial species has justifiably alarmed the medical and research communities. Frequently, resistant

organisms are co-resistant to several antibacterial agents. Penicillin resistance in *S. pneumoniae* has been particularly problematic. This organism causes upper respiratory tract infections. Modification of a penicillin-binding protein (PBP) underlies resistance to penicillin in the majority of cases. Combatting resistance to antibiotic agents will require research into the molecular biology of pathogenic organisms. The goal of such research will be to identify new antibacterial agents.

In the meanwhile, researchers continue to develop antibiotics effective against a number of microorganisms. Particularly problematic are refractory microorganisms, e.g., *S. pneumoniae*, that are highly recombinogenic and that readily take up exogenous DNA from their surroundings. Thus, there is a need for new antibacterial compounds and new targets for antibacterial therapy against microorganisms, particularly with respect to minimal gene set genes thereof.

Summary of the Invention

Accordingly, in a first aspect, the present invention provides an Era/RNA complex, comprising:

at least one Era protein; and

at least one RNA species selected from the group consisting of 16S rRNA, 23S rRNA, 5S rRNA, mRNA of Era, an oligomeric fragment of any one of the foregoing RNA species, a probe derived from any one of the foregoing RNA species, a primer derived from any one of the foregoing RNA species, and mixtures thereof. Preferred RNA species are 16S rRNA and 23S rRNA. 16S rRNA can be the predominant RNA species present in the Era/RNA complex.

The oligomeric fragment can comprise a portion of the RNA species, or it can be synthesized from the RNA used as a template, or synthesized based on knowledge of the RNA sequence. The length of such probes or primers can be in the range of from about 6 nucleotides to about 2,900 nucleotides. It should be noted that the probe or primer can be RNA or DNA. It should also be noted that DNA can be present in the complex, alone or in combination with RNA. Either or both of the RNA and DNA can be of procaryotic,

e.g., bacterial, or eucaryotic origin. In any case, such RNA or DNA should possess the property that it forms a complex with the Era protein. The Era protein and the RNA (and DNA) species can be obtained from the same or different biological source, either or both of which can be procaryotic or eucaryotic. For example, the Era protein and at least one RNA species are obtainable from a microorganism selected from the group consisting of *Streptococcus sp.*, *Escherichia sp.*, *Staphylococcus sp.*, *Haemophilus sp.*, *Mycoplasma sp.*, *Mycobacteria sp.*, *Enterococcus sp.*, *Chlamydia* sp., a mutant of any of the foregoing, and progeny of any of the foregoing, preferably from *Streptococcus sp.*, *Escherichia sp.*, a mutant of either of the foregoing, or progeny of either of the foregoing. Preferred species include, but are not limited to, *Streptococcus pneumoniae, Escherichia coli, Staphylococcus aureus, Haemophilus influenzae, Mycoplasma pneumoniae, Mycobacteria tuberculosis, Enterococcus feacium, Chlamydia pneumoniae*, a mutant of any of the foregoing, and progeny of any of the foregoing. More preferred species include *Streptococcus pneumoniae*, *Escherichia coli* , a mutant of either of the foregoing, and progeny of any of the foregoing.

In a second aspect, the present invention provides a method of producing the Era/RNA complex *in vitro*, comprising effecting *in vitro* the formation of a complex between at least one Era protein and at least one RNA species selected from the group consisting of 16S rRNA, 23S rRNA, 5S rRNA, mRNA of Era, an oligomeric fragment of any one of the foregoing RNA species, a probe derived from any one of the foregoing RNA species, a primer derived from any one of the foregoing RNA species, and mixtures thereof.

In a third aspect, the present invention provides a method of producing the Era/RNA complex *in vivo*, comprising expressing at least one Era protein in a cell containing at least one RNA species selected from the group consisting of 16S rRNA, 23S rRNA, 5S rRNA, and mRNA of Era, and recovering the Era/RNA complex. In this method, the Era protein can be homologous or heterologous to the host cell, which can be procaryotic, e.g., bacterial, or eucaryotic. Similarly, the RNA can be endogenous, or transcribed from introduced DNA heterologous to the host cell. In this method, the Era protein can be overexperessed, and the Era/RNA complex can be isolated

electrophoretically, for example by agarose or polyacrylamide gel electrophoresis, or chromatographically, for example by gel filtration column chromatography, e.g., Superdex column chromatography; affinity chromatography, e.g., glutathione column, Nickel column chromatography, or antibody column chromatography; ion exchange chromatography, e.g., Q-column chromatography (anion-exchange) or S-column chromatography (cation exchange); adsorption chromatography, e.g., hydroxyapatite column chromatography; hydrophobic interaction chromatography, e.g., phenyl-Separose chromatography; or dye chromatography, e.g., Reactive Blue chromatography.

In another aspect, the present invention provides a method of identifying a compound that inhibits the formation of an Era/RNA complex of the present invention, wherein the Era protein or the RNA species thereof is independently optionally labeled, comprising:

a) determining the amount of Era/RNA complex formed in the absence of the compound; and

b) comparing the amount of Era/RNA complex formed in a) with the amount of Era/RNA complex formed in the presence of the compound,

wherein any reduction in the amount of Era/RNA complex formed in b) compared to that formed in a) indicates that the compound inhibits the formation of the Era/RNA complex.

In this method, at least one of the Era protein or the RNA species, or both, can be labeled using a radiolabel, a fluorescent tag, or biotin/avidin. The amount of Era/RNA complex formed in steps a) and b) can be determined electrophoretically. Furthermore, formation of the Era/RNA complex can be achieved in several ways: by adding the compound to a mixture of the Era protein and the RNA species; by adding the RNA species to a mixture of the Era protein and the compound; or by adding the Era protein to a mixture of the RNA species and the compound.

In another aspect, the present invention provides a method of identifying a compound that decreases the stability of the Era/RNA complex, wherein the Era protein

or the RNA species thereof can be independently optionally labeled using a radiolabel, a fluorescent tag, or biotin/avidin, comprising:

a) determining the stability of the Era/RNA complex in the absence of the compound; and

b) comparing the stability of the Era/RNA complex in a) with the stability of the Era/RNA complex formed in the presence of the compound,

wherein any reduction in the stability of the Era/RNA complex in b) compared to that in a) indicates that the compound decreases the stability of the Era/RNA complex. The stability of the Era/RNA complex can be determined by scintillation proximity assay or electrophoresis of aliquots of reaction mixture over time.

In another aspect, the present invention provides a method of identifying a compound that binds to the Era/RNA complex, wherein the Era protein or the RNA species thereof is independently optionally labeled, comprising contacting the Era/RNA complex and the compound, and measuring the binding of the compound to the Era/RNA complex.

In another aspect, the present invention provides a method of identifying a compound that binds to the Era/RNA complex, wherein the Era protein or the RNA species thereof is independently optionally labeled, comprising adding the compound to a mixture of the Era protein and the RNA species, and measuring the binding of the compound to Era/RNA complex formed.

In another aspect, the present invention provides a method of identifying a compound that binds to the Era/RNA complex, wherein the Era protein or the RNA species thereof is independently optionally labeled, comprising adding the RNA species to a mixture of the Era protein and the compound, and measuring the binding of the compound to Era/RNA complex formed.

In yet another aspect, the present invention provides a method of Identifying a compound that binds to the Era/RNA complex, wherein the Era

protein or the RNA species thereof is independently optionally labeled, comprising adding the Era protein to a mixture of the RNA species and the compound, and measuring the binding of the compound Era/RNA complex formed.

In yet another aspect, the present invention provides a method of identifying a compound that inhibits the enzymatic or ligand binding activity of Era protein, comprising:

a) determining the enzymatic or ligand binding activity of said Era protein in the absence of the compound; and

b) comparing the enzymatic or ligand binding activity of the Era protein in a) with the enzymatic or ligand binding activity of the Era protein in the presence of the compound,

wherein any reduction in the enzymatic or ligand binding activity of the Era protein in b) compared to that in a) indicates that the compound inhibits the enzymatic or ligand binding activity of the Era protein.

In this method, the enzymatic or ligand binding activity of the Era protein can be selected from Era protein GTPase hydrolysis activity, Era protein GTP binding activity, and Era protein GDP binding activity.

In a still further aspect, the present invention provides a method such as the foregoing, but wherein the determining of steps a) and b) comprises measuring the enzymatic or ligand binding activity of Era protein wherein the Era protein is in the form of an Era/RNA complex.

In yet another aspect, the present invention provides a method of identifying a compound that inhibits the enzymatic or ligand binding activity of Era protein, comprising:

a) determining the enzymatic or ligand binding activity of the Era protein in the absence of the compound and the presence of acetate or 3-phosphoglycerate; and

b) comparing the enzymatic or ligand binding activity of the Era protein in a) with the enzymatic or ligand binding activity of the Era protein in the presence of acetate or 3-phosphoglycerate and the compound,

wherein any reduction in the enzymatic or ligand binding activity of the Era protein in b) compared to that in a) indicates that the compound inhibits the enzymatic or ligand binding activity of the Era protein.

Further scope of the applicability of the present invention will become apparent from the detailed description provided below. However, it should be understood that the detailed description and specific examples, while indicating preferred embodiments of the present invention, are given by way of illustration only since various changes and modifications within the spirit and scope of the invention will become apparent to those skilled in the art from this detailed description.

Detailed Description of the Invention

The following detailed description of the invention is provided to aid those skilled in the in practicing the present invention. Even so, the following detailed description should not be construed to unduly limit the present invention as modifications and variations in the embodiments discussed herein can be made by those of ordinary skill in the art without departing from the spirit or scope of the present inventive discovery.

The contents of the references cited herein are herein incorporated by reference in their entirety.

Conventional methods of gene isolation, molecular cloning, vector construction, etc., are well known in the art and are summarized in Sambrook et al. (1989) *Molecular Cloning: A Laboratory Manual*, Second Edition, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, and Ausubel et al. (1989) *Current Protocols in Molecular Biology*, John Wiley & Sons, Inc. One skilled in the art can readily reproduce the plasmid vectors described herein without undue experimentation employing these methods in conjunction with the cloning information provided herein. The various DNA

sequences, fragments, etc., necessary for this purpose can be readily obtained as components of commercially available plasmids, or are otherwise well known in the art and publicly available.

As used above, and throughout the following description of the invention, the following terms, unless otherwise indicated, shall be understood to have the following meanings:

As used herein, "Era" refers to the Era protein from microorganisms including, but not limited to, *Streptococcus sp.*, e.g., *Streptococcus pneumoniae; Escherichia sp.*, e.g., *Escherichia coli*; *Staphylococcus sp.*, e.g., *Staphylococcus aureus; Haemophilus sp.*, e.g., *Haemophilus influenzae;, Mycoplasma sp.*, e.g., *Mycoplasma pneumoniae; Mycobacteria sp.*, e.g., *Mycobacteria tuberculosis; Enterococcus sp.*, e.g., *Enterococcus feacium; Chlamydia* sp., e.g., *Chlamydia pneumoniae*, or any mutant or progeny of any of the foregoing. Particular sources of the Era protein include *Streptococcus sp.*, e.g., *Streptococcus pneumoniae*, and *Escherichia sp.*, e.g., *Escherichia coli.*

The *era* gene from *S. pneumoniae* has the nucleic acid sequence shown in SEQ ID NO:1 herein. The present invention encompasses nucleic acid fragments and sequences comprising SEQ ID NO:1, as well as sequences consisting essentially of, and consisting of, this sequence. The Era protein from *S. pneumoniae* has the amino acid sequence also shown in SEQ ID NO:2 herein. The present invention encompasses amino acid fragments and sequences comprising SEQ ID NO:2, as well as sequences consisting essentially of, and consisting of, this sequence.

SEQ ID NO:1:

```
ATG ACT TTT AAA TCA GGC TTT GTA GCC ATT TTA GGA CGT CCC AAT GTT
48
Met Thr Phe Lys Ser Gly Phe Val Ala Ile Leu Gly Arg Pro Asn Val
  1               5                  10                  15

GGG AAG TCA ACC TTT TTA AAT CAC GTT ATG GGG CAA AAG ATT GCC ATC
96
Gly Lys Ser Thr Phe Leu Asn His Val Met Gly Gln Lys Ile Ala Ile
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                20                  25                  30

ATG AGT GAC AAG GCG CAG ACA ACG CGC AAT AAA ATC ATG GGA ATT TAC
144
Met Ser Asp Lys Ala Gln Thr Thr Arg Asn Lys Ile Met Gly Ile Tyr
          35                  40                  45

ACG ACT GAT AAG GAG CAA ATT GTC TTT ATC GAC ACA CCA GGG ATT CAC
192
Thr Thr Asp Lys Glu Gln Ile Val Phe Ile Asp Thr Pro Gly Ile His
      50                  55                  60

AAA CCT AAA ACA GCT CTC GGA GAT TTC ATG GTT GAG TCT GCC TAC AGT
240
Lys Pro Lys Thr Ala Leu Gly Asp Phe Met Val Glu Ser Ala Tyr Ser
 65                  70                  75                  80
ACC CTT CGC GAA GTG GAC ACT GTT CTT TTC ATG GTG CCT GCT GAT GAA
288
Thr Leu Arg Glu Val Asp Thr Val Leu Phe Met Val Pro Ala Asp Glu
                 85                  90                  95

GCG CGT GGT AAG GGG GAC GAT ATG ATT ATC GAG CGT CTC AAG GCT GCC
336
Ala Arg Gly Lys Gly Asp Asp Met Ile Ile Glu Arg Leu Lys Ala Ala
            100                 105                 110

AAG GTT CCT GTG ATT TTG GTG GTG AAT AAA ATC GAT AAG GTC CAT CCA
384
Lys Val Pro Val Ile Leu Val Val Asn Lys Ile Asp Lys Val His Pro
        115                 120                 125

GAC CAG CTC TTG TCT CAG ATT GAT GAC TTC CGT AAT CAA ATG GAC TTT
432
Asp Gln Leu Leu Ser Gln Ile Asp Asp Phe Arg Asn Gln Met Asp Phe
    130                 135                 140

AAG GAA ATT GTT CCA ATC TCA GCC CTT CAG GGA AAT AAC GTG TCT CGT
480
Lys Glu Ile Val Pro Ile Ser Ala Leu Gln Gly Asn Asn Val Ser Arg
145                 150                 155                 160
CTA GTG GAT ATT TTG AGT GAA AAT CTG GAT GAA GGT TTC CAA TAT TTC
528
Leu Val Asp Ile Leu Ser Glu Asn Leu Asp Glu Gly Phe Gln Tyr Phe
                165                 170                 175
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CCG TCT GAT CAA ATC ACA GAT CAT CCA GAA CGT TTC TTA GTT TCA GAA
576
Pro Ser Asp Gln Ile Thr Asp His Pro Glu Arg Phe Leu Val Ser Glu
            180                 185                 190

ATG GTT CGC GAG AAA GTC TTG CAC CTA ACT CGT GAA GAG ATT CCG CAT
624
Met Val Arg Glu Lys Val Leu His Leu Thr Arg Glu Glu Ile Pro His
        195                 200                 205

TCT GTA GCA GTA GTT GTT GAC TCT ATG AAA CGA GAC GAA GAG ACA GAC
672
Ser Val Ala Val Val Val Asp Ser Met Lys Arg Asp Glu Glu Thr Asp
    210                 215                 220

AAG GTT CAC ATC CGT GCA ACC ATC ATG GTC GAG CGC GAT AGC CAA AAA
720
Lys Val His Ile Arg Ala Thr Ile Met Val Glu Arg Asp Ser Gln Lys
225                 230                 235                 240

GGG ATT ATC ATC GGT AAA GGT GGC GCT ATG CTT AAG AAA ATC GGT AGT
768
Gly Ile Ile Ile Gly Lys Gly Gly Ala Met Leu Lys Lys Ile Gly Ser
                245                 250                 255

ATG GCC CGT CGT GAT ATC GAA CTC ATG CTA GGA GAC AAG GTC TTC CTA
816
Met Ala Arg Arg Asp Ile Glu Leu Met Leu Gly Asp Lys Val Phe Leu
            260                 265                 270

GAA ACC TGG GTC AAG GTC AAG AAA AAC TGG CGC GAT AAA AAG CTA GAT
864
Glu Thr Trp Val Lys Val Lys Lys Asn Trp Arg Asp Lys Lys Leu Asp
        275                 280                 285

TTG GCT GAC TTT GGC TAT AAT GAA AGA GAA TAC TAA
900
Leu Ala Asp Phe Gly Tyr Asn Glu Arg Glu Tyr
    290                 295
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SEQ ID NO:2:

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Met Thr Phe Lys Ser Gly Phe Val Ala Ile Leu Gly Arg Pro Asn Val
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1               5                  10                  15
Gly Lys Ser Thr Phe Leu Asn His Val Met Gly Gln Lys Ile Ala Ile
            20                  25                  30
Met Ser Asp Lys Ala Gln Thr Thr Arg Asn Lys Ile Met Gly Ile Tyr
        35                  40                  45
Thr Thr Asp Lys Glu Gln Ile Val Phe Ile Asp Thr Pro Gly Ile His
    50                  55                  60
Lys Pro Lys Thr Ala Leu Gly Asp Phe Met Val Glu Ser Ala Tyr Ser
65                  70                  75                  80
Thr Leu Arg Glu Val Asp Thr Val Leu Phe Met Val Pro Ala Asp Glu
                85                  90                  95
Ala Arg Gly Lys Gly Asp Asp Met Ile Ile Glu Arg Leu Lys Ala Ala
            100                 105                 110
Lys Val Pro Val Ile Leu Val Val Asn Lys Ile Asp Lys Val His Pro
        115                 120                 125
Asp Gln Leu Leu Ser Gln Ile Asp Asp Phe Arg Asn Gln Met Asp Phe
    130                 135                 140
Lys Glu Ile Val Pro Ile Ser Ala Leu Gln Gly Asn Asn Val Ser Arg
145                 150                 155                 160
Leu Val Asp Ile Leu Ser Glu Asn Leu Asp Glu Gly Phe Gln Tyr Phe
                165                 170                 175
Pro Ser Asp Gln Ile Thr Asp His Pro Glu Arg Phe Leu Val Ser Glu
            180                 185                 190
Met Val Arg Glu Lys Val Leu His Leu Thr Arg Glu Glu Ile Pro His
        195                 200                 205
Ser Val Ala Val Val Val Asp Ser Met Lys Arg Asp Glu Glu Thr Asp
    210                 215                 220
Lys Val His Ile Arg Ala Thr Ile Met Val Glu Arg Asp Ser Gln Lys
225                 230                 235                 240
Gly Ile Ile Ile Gly Lys Gly Gly Ala Met Leu Lys Lys Ile Gly Ser
                245                 250                 255
Met Ala Arg Arg Asp Ile Glu Leu Met Leu Gly Asp Lys Val Phe Leu
            260                 265                 270
Glu Thr Trp Val Lys Val Lys Lys Asn Trp Arg Asp Lys Lys Leu Asp
        275                 280                 285
Leu Ala Asp Phe Gly Tyr Asn Glu Arg Glu Tyr
    290                 295
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The amino acid sequences of Era proteins from other organisms are known in the art. For example, the *E. coli* Era protein amino acid sequence is described by Ahnn, J., *et al.*, Proc. Natl. Acad. Sci. USA, 83, 8849-8853 (1986); Britton, R.A., et al, J. Bacteriol., 179, 4575-4582 (1997); and Chen, S.-M. et al, J. Biol. Chem., 265, 2888-2895 (1990).

The *Haemophilus influenzae* Era protein amino acid sequence is described by Fleischmann *et al.*, Science, 269:496-512 (1995). The *M. tuberculosis* Era protein amino acid sequence is described by Cole *et al.*, Nature, 393, 537-544 (1998), and the *M. pneumoniae* Era protein amino acid sequence is described by Himmelreich *et al.*, Nucleic Acids Res., 24, 4420-4449 (1996). Using techniques known to those skilled in art, e.g., Southern hybridization, the PCR, and functional complementation of, for example, *E. coli era* mutants, one can identify and isolate other *era* genes from other organisms where these gene sequences are similar to known *era* genes.

The term "Era/RNA complex refers to a complex resulting from an interaction, e.g., covalent binding, or non-covalent binding, e.g., hydrogen bonding or van derWaals interactions, between the Era protein from a eukaryote or prokaryotic microorganism, or any mutant or progeny thereof, and at least one or more RNA sequences selected from the following: 16S rRNA, 23S rRNA, 5S rRNA, mRNA of Era, or a probe or primer derived from any of these RNAs. The probe or primer can span only a portion or the entire length of any of the foregoing RNA sequences and can range in length from about 6-18 nucleotides to about 3,000 nucleotides, preferably from about 100 nucleotides to about 3,000 nucleotides, more preferably from about 120 nucleotides to about 2900 nucleotides. It should be noted that the present invention encompasses not only Era/RNA complexes, but Era/nucleic acid complexes including Era/DNA complexes as well.

A "primer" is a nucleic acid fragment that functions as an initiating substrate for enzymatic or synthetic elongation of, for example, a nucleic acid molecule.

A "probe" is a labeled nucleic acid fragment (or unlabeled counterpart thereof) that can hybridize with another nucleic acid compound or fragment thereof.

Skilled artisans will recognize that the DNA probes or primers, or fragments thereof, of the present invention can be generated by general cloning methods. PCR amplification using oligonucleotide primers targeted to any suitable region of SEQ ID NO:1 is preferred. Methods for PCR amplification are widely known in the art. See, e.g., PCR Protocols: A Guide to Method and Application, Ed. M. Innis, *et al.*, Academic

Press (1990) or U.S. Patent No. 4,889,818, which is herein incorporated by reference. A PCR comprises DNA, suitable enzymes, primers, and buffers, and is conveniently carried out in a DNA Thermal Cycler (Perkin Elmer Cetus, Norwalk, CT). A positive PCR result is determined by, for example, detecting an appropriately-sized DNA fragment following agarose gel electrophoresis.

The DNA and RNA probes or primers, or fragments thereof, can also be produced using synthetic methods known in the art. See, e.g., E.L. Brown, *et al.*, *Methods in Enzymology*, 68 109-151 (1979). An apparatus such as the Applied Biosystems Model 380A or 380B DNA synthesizers (Applied Biosystems, Inc., 850 Lincoln Center Drive, Foster City, CA 94404) can be used to synthesize DNA. Synthetic methods rely upon phosphotriester chemistry (See, e.g., M.J. Gait, ed., *Oligonucleotide Synthesis, A Practical Approach*, (1984)), or phosphoramidite chemistry.

"Substantially purified" means a specific isolated nucleic acid fragment or protein of the present invention, or fragment thereof, in which substantially all contaminants (*i.e.*, substances that differ from said nucleic acid fragment, protein, or fragment thereof) have been removed from said nucleic acid fragment, protein, or fragment thereof. A substantially purified nucleic acid fragment, protein, or fragment thereof of the present invention is at least about 75%, preferably at least about 80%, more preferably at least about 85%, more preferably at least about 90%, even more preferably at least about 95%, even more preferably at least about 99%, and even more preferably still greater than 99% pure. For example, a protein may, but not necessarily, be "substantially purified" by the IMAC method as described hereinbelow.

"Consensus sequence" refers to an amino acid or nucleotide sequence that may suggest the biological function of a protein, DNA, or RNA molecule. Consensus sequences are identified by comparing proteins, RNAs, and gene homologues from different species.

The term "cleavage" or "restriction" of DNA refers to the catalytic cleavage of the DNA with a restriction enzyme that acts only at certain sequences in the DNA (viz.

sequence-specific endonucleases). The various restriction enzymes used herein are commercially available and their reaction conditions, cofactors, and other requirements are used in the manner well known to one of ordinary skill in the art. Appropriate buffers and substrate amounts for particular restriction enzymes are specified by the manufacturer or can readily be found in the literature.

The term "plasmid" refers to an extrachromosomal genetic element (Sambrook, J., *et al.*, *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y., (1989)). The starting plasmids herein are either commercially available, publicly available on an unrestricted basis, or can be constructed from available plasmids in accordance with published procedures. In addition, equivalent plasmids to those described are known in the art and will be apparent to the ordinarily skilled artisan.

"Recombinant DNA cloning vector" (Sambrook, J., *et al.*, *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y., (1989)) as used herein refers to any autonomously replicating agent, including, but not limited to, plasmids and phages, comprising a DNA molecule to which one or more additional DNA segments can or have been added.

The term "vector" (Sambrook, J., *et al.*, *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y., (1989)) as used herein refers to a nucleic acid compound used for introducing exogenous DNA into host cells. A vector comprises a nucleotide sequence that may encode one or more protein molecules. Plasmids, cosmids, viruses, and bacteriophages, in the natural state or which have undergone recombinant engineering, are examples of commonly used vectors.

The terms "complementary" or "complementarity" as used herein refer to the capacity of purine and pyrimidine nucleotides to associate through hydrogen bonding to form double stranded nucleic acid molecules. The following base pairs are related by complementarity: guanine and cytosine; adenine and thymine; and adenine and uracil. As used herein, "fully complementary" refers to all base pairs comprising two single-stranded nucleic acid molecules. "Partially complementary" refers to the condition in

which one of two complementary single-stranded nucleic acid molecules is shorter than the other, such that one of the molecules remains partially single-stranded.

"Selective hybridization" refers to hybridization under conditions of high stringency. The degree of hybridization between nucleic acid molecules depends upon, for example, the degree of complementarity, the stringency of hybridization, and the length of hybridizing strands.

The term "stringency" (Sambrook, J., *et al.*, *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y., (1989)) relates to nucleic acid hybridization conditions. High stringency conditions disfavor non-homologous base pairing. Low stringency conditions have the opposite effect. Stringency may be altered, for example, by changes in temperature and/or formamide and/or salt concentration. Typical high stringency conditions comprise hybridizing at about 50°C to about 65°C in about 5X SSPE and about 50% formamide, and washing at about 50°C to about 65°C in about 0.5X SSPE. Typical low stringency conditions comprise hybridizing at about 35°C to about 37°C in about 5X SSPE and about 40% to about 45% formamide, and washing at about 42°C in about 1X-2X SSPE.

"SSPE" denotes a hybridization and wash solution comprising sodium chloride, sodium phosphate, and EDTA, at pH 7.4. A 20X solution of SSPE is made by dissolving 174 g of NaCl, 27.6 g of $NaH_2PO4{\cdot}H_2O$, and 7.4 g of EDTA in 800 ml of H_2O. The pH is adjusted with NaOH, and the volume is brought to 1 liter.

"SSC" denotes a hybridization and wash solution comprising sodium chloride and sodium citrate at pH 7. Dissolving 175g of NaCl and 88 g of sodium citrate in 800ml of H_2O makes a 20X solution of SSC. The volume is brought to 1 liter after adjusting the pH with 10N NaOH.

The term "about" or "approximately" means within 20%, preferably within 15%, more preferably within 10%, and even more preferably within 5%, of a given value or range.

It is to be understood that this invention covers all appropriate combinations of the particular and preferred groupings referred to herein.

Exemplary methods useful in practicing the present invention include, but are not limited to, the following:

Bacterial Strains and Culture Conditions

The following *E. coli* strains are used in this study: *E. coli* K-12, a wild type strain (Zhao, G., & Winkler, M. E., *J. Bacteriol.* 177, 883-891 (1995)), *E. coli* BL21 (DE3) plysS [F^- *dcm ompT hsdS* (r_B-m_B-) *gal* λ (DE3) (plysS, cam^r)] (Stratagene, La Jolla, CA), *E. coli* XL1 Blue [MRF', *rec1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac(F' proAB lacI^q ZΔM15)*] (Stratagene), LY66 [*E. coli* XL1 Blue/pRBP-13 (*GST-era*$^+$, amp^r)], LY68[*E. coli* BL21 (DE3) plysS/pRBP-11 (*era*$^+$, amp^r)], LY70 [*E. coli* BL21 (DE3) plysS/pRBP-12 (*His-era*$^+$, amp^r)], LY52 [*E. coli* XL1 Blue/pLY52 (*GST-Δera*$^+$, amp^r)] Zhao, G., *et al.*, *Microbiology* 145, 791-800 (1999), LY41 (*E. coli* XL1 Blue/pLY41 (*GST-era*$^+$, amp^r) Zhao, G., *et al.*, *Microbiology* 145, 791-800 (1999), and LY160 [*E. coli* XL1 Blue/pGEX-2T (*GST*$^+$, amp^r)] (Pharmacia LKB Biotechnology, Alameda, CA). These strains are useful in expressing Era from *E. coli* or other organisms.

Cloning of the *era* gene from *E. coli*

The *E. coli era* gene is directly cloned by PCR amplification of the chromosomal DNA of *E. coli* (Zhao, G., *et al.*, *Microbiology* 145, 791-800 (1999)) by using the two primers designed based on the published sequence (Ahnn, J., *et al.*, *Proc. Natl. Acad. Sci. USA*, 83, 8849-8853 (1986); Britton, R. A., *et al.*, *J. Bacteriol.*, 179, 4575-4582 (1997), Britton, R. A., *et al.*, *Mol. Microbiol.*, 27, 739-750 (1998)). The 5' PCR primer (5'CCGGAATTCAGATCTCATATGAGCATCGATAAAAGTTAC-3') (SEQ ID NO:3)

is designed at the ATG start codon of *era*, and contains *Eco*RI , *Bgl*II, and *Nde*I sites for cloning purposes. The 3' PCR primer (5'-CCGGAATTCAGATCTTTAAAGATCGTCAACGTAACCGAG-3') (SEQ ID NO:4) is designed at the stop codon of *era*, and contains *Eco*RI and *Bgl*II sites after the stop codon. Using these primers, the *era* gene is PCR amplified from *E. coli* as described (Zhao, G., *et al.*, Microbiology 145, 791-800 (1999)) under the following conditions: denaturation at 94°C for 30 sec, annealing at 60°C for 30 sec, and polymerization at 72°C for 30 sec for 25 cycles. Five PCR reaction products are combined, and a portion of the pooled PCR products is digested with *Eco*RI. The *Eco*RI digested PCR fragment is cloned into pBCKS+ (Stratagene, La Jolla, CA) previously digested with *Eco*RI and dephosphorylated with calf intestinal alkaline phosphatase (Gibco BRL). *era* from several pBCKS+ clones is sequenced and a clone containing the consensus *era* gene sequence (pRBP-10) is used for constructing expression plasmids. pRBP-10 is partially digested with *Nde*I, and then *Bgl*II. The *Nde*I-*Bgl*II DNA fragment containing *era* is subcloned into pET-11a and pET-15b, containing a his tag, at the *Nde*I and *Bam*HI sites (Novagen, Madison, WI). The resulting constructs are designated as pRBP-11 and pRBP-12, respectively. pRBP-10 is also digested with *Bgl*II, and the *Bgl*II fragment of *era* is subcloned into pGEX-2T (Pharmacia) at the *Bam*HI site. Plasmid pGEX-2T comprises a GST sequence and a thrombin cleavage site that facilitates production of a GST-Era fusion protein which can be cleaved by thrombin. The resulting construct is designated as pRBP-13.

Cultures comprising cells containing native expressed Era or cells containing plasmids comprising the Era gene and a glutathione S-transferase (GST)-Era fusion gene containing a thrombin cleavage site are prepared and grown substantially as described in the Protein Preparation Methods Section, below. For expression of Era, all *E. coli* strains are first grown overnight at 35°C with vigorous shaking in LB medium (Bio 101, Inc., La Jolla, CA) supplemented with 100 μg ampicillin (Amp) per ml. The overnight cultures (approximately 40 mls) are inoculated into 1.25 liters each of fresh LB medium (Bio 101) containing Amp, and then induced at an optical density of 0.5 to 0.6 at 600 nm (OD_{600}) with 0.8 mM IPTG (Gibco BRL, Gaithersburg, MD) for 3 hr at 33°C. Cells are harvested by centrifugation at 4,000 x *g* for 10 min, and washed with 20 mM Tris-HCl, pH 8.0, and

5 mM $MgCl_2$. *E. coli* K-12 (a wild type strain) is grown overnight in LB at 35°C with vigorous shaking. The overnight culture (approximately 40 mls) is inoculated into 250 ml of fresh LB medium. The culture is harvested at OD_{600} = 0.8-1.00 by centrifugation as described above for recovery of Era protein.

When Era protein is purified from cells, an Era/RNA complex is obtained. The presence of the complex has been confirmed by gel filtration column chromatography and agarose gel electrophoresis; the complex is present as a very high molecular weight species that is significantly larger than Era alone. The Era protein is isolated as follows.

Purification of GST-Era Protein

The culture cells containing a plasmid that carries a GST fusion Era gene are re-suspended in 20mM Tris, pH 7.5, 140mM NaCl, and 5mM $MgCl_2$ (buffer A). The resulting suspension is disrupted by passing twice through a 20K French press cell (Aminco Laboratories, Inc., Rochester, NY) and centrifuged at 180,000*g* for 45 min. The supernatant collected is loaded onto a 10ml glutathione-Sepharose column (Pharmacia Biotechnology). The column is washed with 60ml of buffer A and eluted with 10mM glutathione in buffer A. All fractions collected are subjected to SDS-PAGE analysis (Laemmli, U. K. *Nature (London)* 227, 680-685 (1970)), and those containing GST-Era are collected and dialyzed (molecular mass cut-off, 25kDa, Sigma) in 4 liters of 20mM Tris, pH 8.0, 5mM $MgCl_2$ (buffer B) at 4°C overnight. After dialysis, glycerol is added to the dialyzed Era preparation at a final concentration of 16% (v/v), and the enzyme preparation is aliquoted and stored at -70°C. Protein concentration is determined using a Bradford protein assay kit (Bio-Rad) with BSA as a standard (Bradford, M. M., *Anal. Biochem.* 72, 248-254 (1976)).

Purification of Native Form of Era Protein

The culture cells containing a plasmid that carries the Era gene are grown, collected, and disrupted as described above. The resulting suspension is centrifuged as described above, and the supernatant is loaded onto a Source Q (30μM diameter) column

(2.6 by 10cm) that is previously equilibrated with buffer B. The column is washed with 250ml of buffer B and eluted with a linear gradient of 0-1,000mM KCl in buffer B. The presence of Era in the fractions is monitored by SDS-PAGE (Laemmli, U. K. *Nature (London)* 227, 680-685 (1970)). The fractions containing Era are pooled and dialyzed as described above against 4 liters (no change) of 20mM potassium phosphate, pH 7.5. The dialyzed enzyme preparation is applied to a Macro-Prep ceramic hydroxyapatite type I (80μM diameter) column (2.6 by 10cm) that is equilibrated with 20mM potassium phosphate, pH 7.5. The column is washed with 250ml of the buffer and eluted with a linear gradient of 20-700mM potassium phosphate, pH 7.5. The fractions containing Era are pooled and dialyzed as described above in buffer B and loaded onto a Heparin (Bio-Rad) column (2.6 by 10 cm) that is equilibrated with buffer B. The column is washed with 250ml of buffer B and eluted with a linear gradient of 0-1,000mM KCl in buffer B. The fractions containing Era are pooled and dialyzed as described above in buffer B. The purified Era protein is aliquoted and stored as described above. Protein concentration is determined using a Bradford protein assay kit (Bio-Rad) with BSA as a standard (Bradford, M. M., *Anal. Biochem.* 72, 248-254 (1976)).

For purification of His-tagged Era, culture cells are resuspended in 20 mM Tris-HCl, pH 8.0, 5 mM $MgCl_2$ and 0.5 M NaCl (buffer C). The resulting suspension is disrupted and centrifuged as described above. The supernatant fraction collected is loaded onto a 10 ml chelating Sepharose column charged with 50 mM $NiCl_2$. The column is washed with 60 ml of buffer C and then 60 mM imidazole in buffer C, and eluted with 0.5 M imidazole in buffer C. Fractions containing His-Era are collected and dialyzed as described above

The Era/RNA complex obtained exhibits the following properties, characteristic of RNA: 1) maximal absorption at 260 nm; 2) sensitivity to RNase digestion; and 3) ethidium bromide staining, as described below.

Antibody Preparation

Polyclonal antibodies against SDS-denatured, full-length native Era protein of *E. coli* or *S. pneumoniae* are prepared (Robert Sargent, Inc., Ramona, CA). A purified Era protein preparation is denatured using SDS and subjected to SDS-PAGE (10% gel, Bio-Rad) as described by Laemmli, U. K. *Nature (London)* 227, 680-685 (1970). Protein bands are visualized by incubating the gel in a solution containing 0.5 M KCl and 50 mM potassium phosphate (pH 7.2), and excised (Zhao, G., & Winkler, M. E., *J. Bacteriol.* 177, 883-891 (1995)). Each protein band, which contains approximately 100 μg of Era, is injected into two New Zealand white rabbits. Antibodies are obtained as described by Zhao et al., *Microbiology* 145:791-800 (1999).

Demonstration of RNA association with Era protein

To determine if Era isolated directly from a wild-type *E. coli* or *S. pneumoniae* strain (Zhao, G., & Winkler, M. E., *J. Bacteriol.* 177, 883-891 (1995)) is also associated with RNA, a crude extract of either strain is prepared in buffer A as described above, and subjected to gel filtration column chromatography (HiLoad 16/60 Superdex 200 column, Pharmacia). To determine the molecular mass value of Era, the column is equilibrated with buffer A and calibrated with the following protein standards (Sigma): blue dextran (2,000 kDa), β-amylase (200 kDa), alcohol dehydrogenase (150 kDa), bovine serum albumin (66 kDa), carbonic anhydrase (29 kDa), and cytochrome C (12.4 kDa), and eluted with buffer A. Fractions (1 ml each) are collected, and 15 μl of each of the fractions are subjected to SDS-PAGE (Laemmli, U. K. *Nature (London)* 227, 680-685 (1970)). The resolved Era protein is transferred to a PVDF membrane and detected by Western blotting analysis as described below using polyclonal antibodies prepared against SDS-denatured native Era protein of *E. coli* or *S. pneumoniae*. Transfer from the gel is carried out in 12 mM Tris-HCl, 96 mM glycine, and 20% methanol (Novex) at room temperature (40 volts, constant) for 2 hr using a Blot Module (Novex). Membranes are blocked in 1x PBS (BRL) containing 5% dry milk at 4°C overnight, incubated with primary antibodies (1/500 dilution) and monoclonal goat anti-rabbit IgG secondary antibodies (Sigma) (1/2000 dilution) for 2-3 hr at room temperature, washed three times with 1x PBS, and developed by using nitroblue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate (Sigma).

Analysis of RNA associated with Era

Purified GST-Era, native Era, and GST proteins (approximately 4 mg each) are extracted with equal volumes of phenol/chloroform/isoamyl alcohol, and the resulting material is collected by ethanol precipitation (Sambrook, J., *et al.*, *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y., (1989)). The pellets (RNA) collected are air dried and resuspended in 200 µl of diethyl pyrocarbonate-treated water, and 10 µl each is analyzed by agarose gel electrophoresis (1.5% agarose containing 0.5 µg ethidium bromide per ml) (Sambrook, J., *et al.*, *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y., (1989)). *E. coli* rRNAs are isolated from a wild-type *E. coli* strain by phenol/chloroform extraction as described (Sambrook *et al.*, 1989).

To directly detect RNA associated with Era, purified Era proteins (approximately 10 µg each) are run on an agarose gel as described above. To determine if RNA is associated with Era proteins, purified Era proteins and phenol-chloroform-extracted material are treated with 1mg RNase A per ml (Boehringer Mannheim, Indianapolis, IN) or 0.2 mg DNase I per ml (Gibco BRL) for 15 min at room temperature and then run on an agarose gel as described above.

Determination of RNA Associated with GST-Era

To determine if nucleic acid is associated with GST-Era, purified GST-Era is loaded onto a Mono Q column (Pharmacia Biotech, Piscataway, NJ) equilibrated with buffer A. The column is washed with buffer A and eluted with a linear gradient of 0-1,000mM KCl in buffer A. Fractions (1ml, each) are collected. Peak fractions containing Era or RNA are treated with RNase A (Sigma) or DNase I (BRL). These fractions are analyzed by agarose gel electrophoresis and spectrophotometric scanning (200-600nm) using a Shimadzu BioSpec-1601 spectrophotometer (Columbia, MD).

To establish that RNA is associated with Era, purified Era proteins (thrombin-treated and untreated GST-Era proteins, and native form of Era) are loaded onto a HiLoad 16/60 Superdex 200 column (Pharmacia) equilibrated with buffer A and eluted with buffer A at a flow rate of 1 ml/min. The molecular mass value of each species resolved is determined by calibrating the column with the following protein standards (Sigma); blue dextran (2,000kDa), alcohol dehydrogenase (150kDa), bovine serum albumin (66kDa), carbonic anhydrase (29kDa), and cytochrome C (12.4kDa). Each protein peak is tested for its GTP hydrolysis and binding activities as described below, and subjected to RNase A and DNase I treatments, agarose gel electrophoresis, and scanning analysis as described above.

Analysis of RNA Associated with Era

Approximately 4 mg each of the GST-Era and native Era proteins are extracted with an equal volume of phenol/chloroform/isoamyl alcohol (25:24:1, v/v, and the aqueous phase is collected after centrifugation (Sambrook, J., *et al.*, *Molecular Cloning: a LaboratoryManual.* Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y. (1989)). Sodium acetate is then added to the aqueous phase at a final concentration of 0.3M followed by adding 2 volumes of 100% ethanol. RNA is precipitated and collected by centrifugation at 14,000*g* for 15 min. The RNA (pellet) collected is air-dried and then re-suspended in 200µl of diethyl pyrocarbonate-treated water. The RNA (10µl, each) is first mixed with 1µl of 10x agarose gel loading buffer (0.4% bromophenol blue, 0.4% xylene cyanol, and 50% glycerol), and then loaded onto a 1% agarose gel containing ethidium bromide (0.5µg/ml). The gel is run in 1x TBE buffer (100mM Tris-HCl, pH 8.4, 90mM boric acid, 1mM EDTA, and 0.5 µg/ml ethidium bromide). RNA is visualized by exposing the gel to an UV transilluminator (254nm).

To directly visualize RNA associated with the protein, approximately 40 µg of purified proteins are placed directly on the gel, as described above.

Purified Era proteins and extracted RNA are treated with 100 units of RNase A (Boehringer Mannheim, Indianapolis, IN) or 20 units of DNase I (Gibco BRL, Gaithersburg, MD) as indicated, for 15 min at room temperature prior to electrophoresis.

Reconstitution of Era Protein and RNA

To reconstitute native Era and GST-Era proteins whose RNA is removed, the proteins are first mixed with extracted RNA in 50mM Tris-HCl, pH 7.5, 5mM $MgCl_2$, and the reaction mixtures are incubated for 0-24 hr at 4, 23, or 37°C. After incubation, GTPase activities are tested using the HPLC method as described below. RNA or RNA oligonucleotides tested included *E. coli* tRNA (Sigma), *E. coli* rRNA (Sigma), RNA extracted from GST-Era, polyA, polyC, polyU, polyG, and polyAGU (Sigma). In addition, phenol/chloroform extracted GST-Era RNA is heat-denatured at 95°C for 5 min, immediately chilled on ice, and then mixed with the proteins, as described above. GTPase activities are tested as described below.

To refold Era protein in the presence of RNA, GST-Era from which RNA is removed by MonoQ column chromatography (Pharmacia) is denatured in 4M and then mixed with phenol/chloroform extracted RNA from GST-Era. The refolding preparation is dialyzed against 4 liters of 50mM Tris-HCl, pH 7.5, and 5mM $MgCl_2$ at 4°C overnight.

It has been determined that RNA bound to Era protein is required for stimulation of Era GTPase activity. Specifically, after the complex is subjected to RNase digestion, the Era protein loses about 10 to about 20 fold GTPase activity. Also, when the RNA bound to Era protein is removed by either salt treatment or column chromatography, the Era protein loses GTPase activity. These data are described below.

Thrombin Cleavage of GST-Era Fusion Protein

To establish that the RNA is specifically associated with the Era part, and not the GST part, of the GST-Era fusion protein, the GST-Era fusion protein is completely cleaved (14 units of thrombin protease/mg of GST-Era in 1x PBS buffer) after overnight

incubation at 4°C. Half of the thrombin-treated protein preparation is subjected to a glutathione Sepharose column to remove the GST-fusion part of the protein. The other half of the protein preparation is not purified further. The resulting preparations are tested for GTPase activity.

Determination of Era GTPase Activity

The GTPase activity of Era is assayed using both thin layer chromatography (TLC) and high pressure liquid chromatography (HPLC) in the presence or absence of acetate or 3-phosphoglycerate. For the TLC assay, reaction mixtures (20 µl, each) contained 0-200mM of acetate or 3-phosphoglycerate, 50mM Tris-HCl, pH 8.0, 5mM $MgCl_2$, 1mM DTT, 100mM NaCl, 1mg/ml BSA, and 10µM of GST-Era. Reactions are initiated by the addition of α-^{32}P-GTP (3000 Ci/mmol, NEN-Dupont), and the reaction mixtures are incubated at 23°C for 60 min for routine assays. After incubation, 2µl of each sample are spotted onto a PEI cellulose thin layer plate (Selecto Scientific, Norcross, Georgia) and dried. The PEI plate is developed in 0.75M KH_2PO_4. The GDP produced is quantified using a Phosphorimage Analyzer (Molecular Dynamics). For the HPLC assay, reaction mixtures (300µl, each) containing 0-200mM of acetate or 3-phosphoglycerate, 50mM Tris-HCl, pH 7.5, 5mM $MgCl_2$, and 1.15µM of GST-Era or native Era are incubated at 23°C or 37°C. To initiate the reactions, GTP is added. Aliquots (100µl, each) are removed after 0 and 30 min incubations, and the reactions are stopped by adding 5µl of 1N HCl. Then, 50µl of each aliquot are injected into a 4.6x250mm ODS-AQ HPLC column (YMC, Inc.), and separated under isocratic conditions (79mM KPO_4, pH 6.0, 4mM tetrabutyl ammonium hydrogen sulfate, 21% methanol). The resulting GDP is quantified by comparing its peak areas with those of GDP standards. Since GDP is bound to the protein when purified, the total amount of GDP produced after 30 min incubation is calculated by subtracting GDP present at the start of the reaction.

The GTPase activity of the Era protein associated with RNA is about 2 to about 30-fold higher than that of Era protein that is not associated with RNA.

When assayed in the presence of acetate or 3-phosphoglycerate, the GTPase activity of Era free of RNA is about 2 to about 30-fold higher than that of Era assayed in the absence of acetate or 3-phosphoglycerate.

Assay of Era GTP and GDP Binding Activity

The k_d of Era for GTP and GDP is determined using a filter binding assay. Reaction mixtures (50µl, each) contained 25 mM Tris-HCl, pH 7.5, 5mM $MgCl_2$, 0.2mg/ml BSA, 1.3µM of GST-Era or native Era, and ^{3}H-GTP (1-100µM) (0.6 Ci/mmol ^{3}H-GTP) or ^{3}H-GDP (1-100µM) (0.6 Ci/mmol ^{3}H-GDP). The reaction mixtures are incubated at room temperature for 30 min and then filtered through a MANP NOB Multiscreen filter plate (Millipore, Bedford, MA). The plate is washed 3 times with 200µl of wash buffer (50mM Tris, pH 7.5, 5mM $MgCl_2$), per well. Then, 30µl of Optiphase Supermix scintillant (Wallac Oy, Finland) is added to each well and the plate is counted using a Wallac Microbeta scintillation counter.

Cloning and Sequence Determination of the RNA Associated with GST-Era Protein of *S. pneumoniae*

The RNA bound to Era protein has been cloned and sequenced. The RNA species that bind to Era protein have been found to include 16S rRNA, 23S rRNA, 5S rRNA, and mRNA of Era protein. The predominant species is 16S rRNA. These experiments are described below. These RNA species can bind alone, or in various combinations, with Era protein.

The synthesis of the first and second strand cDNA of the RNA associated with GST-Era is performed using the SuperScript Choice System for cDNA Synthesis (BRL) following the manufacturer's directions. Two different RNA templates, phenol/chloroform extracted RNA and GST-Era protein that is still associated with RNA, are used for cDNA synthesis. For the first and second strand synthesis, 8µl of extracted RNA or 5µl of GST-Era is mixed with 100ng of random hexamer primers (BRL). After

the second strand synthesis, the DNA fragments are extracted with phenol/chloroform, precipitated with ethanol as described above, and directly used to ligate to pUC18 using the Ready-To-Go pUC18 *SmaI*/BAP$^+$ ligase kit (Pharmacia). The ligation mixtures are then transformed into *E. coli* XL1-Blue MRF (Stratagene). Colonies are randomly picked, and their plasmid DNA is isolated using a Wizard mini prep kit (Promega). DNA sequences of the plasmids isolated are determined using PE-ABI Prism Dye Terminator Cycle Sequencing Ready Reaction fluorescent based chemistry. Sequence data are collected on an ABI377 sequencer (Perkin-Elmer/Applied BioSystems Division) and analyzed using PE-ABI Sequence Analysis v.3.0 software (Perkin-Elmer). Data are edited using Sequencher v.3.0 (Gene Codes Corp.).

Identification and quantitation of the RNA associated with purified GST-Era of *E. coli*

To determine the identity of the RNA associated with the purified GST-Era protein, an affinity-purified GST-Era preparation is subjected to gel filtration column chromatography as described above. A void-volume fraction from the column that contains the GST-Era protein associated with RNA is used as a source of RNA. Total RNA is extracted from 1 ml of the void fraction using an RNeasy midi prep kit (Qiagen, Inc., Valencia, CA), and resuspended in 150 µl of H_2O.

To prepare RNA standards for 16S and 23S rRNA for use in RT-PCR (reverse transcription-PCR), a 500 bp DNA fragment internal to the *E. coli* 16S or 23S rRNA gene is cloned from *E. coli* genomic DNA by PCR as described (Sambrook, J., *et al.*, *Molecular Cloning: a LaboratoryManual.* Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y. (1989); Zhao, G., *et al.*, *Microbiology* 145, 791-800 (1999)) by using the following primers:

5'-CGCGGATCCTGACGTTACCCGCAGAAGAAG-3' (SEQ ID NO:5) and

5'-CCATCGATAAGGTTCTTCGCGTTGCATCG-3' (SEQ ID NO:6) (for 16S rRNA); and

5'-CGCGGATCCTGACCGATAGTGAACCAGTAC-3' (SEQ ID NO:7) and

5'-CCATCGATTCTCCCGTGATAACATTCTCC-3' (SEQ ID NO:8) (for 23S rRNA) based on the published sequences (Brosius, J., *et al.*, Proc. Natl. Acad. Sci. USA 77, 201-204 (1980); Brosius, J., *et al.*, Proc. Natl. Acad. Sci. USA 75, 4801-4805 (1978)). Both 5'-PCR primers contain a *Bam*HI site, and the 3'-PCR primers contain a *Cla*I site for cloning purposes. The amplified DNA fragments are digested with *Bam*HI and *Cla*I and cloned into pBlueScriptII KS+ (Stratagene) that has also been cleaved with both restriction enzymes. The resulting clones containing the DNA fragment corresponding to the 16S or 23S rRNA gene are digested to completion with *Xho*I and 1 μg each of the digested clone is used for *in vitro* transcription by using a MEGscript T7 kit according to the instructions of the manufacturer (Ambion, Inc., Austin, TX). After transcription, the resulting RNA is purified using an RNeasy midi kit (Qiagen). Under these conditions, 123 and 137 μg of RNA is obtained for the clones containing fragments of 16S and 23S rRNA, respectively.

For RT-PCR, all RNA samples are treated with DNaseI (10 μg RNA, 20 units of DNaseI, final volume 200 μl) at 25°C for 15 min. The reaction mixtures are then mixed with 20 μl of 25 mM EDTA and heated to 65°C for 10 min. The final concentration of each RNA preparation is adjusted to 45.5 ng per μl. RT-PCR is performed using a GeneAmp EZ *rTth* RNA PCR kit (Perkin Elmer, Inc.) with the following primers: 5'-TTAACGCGTTAGCTCCGGAAG-3' (SEQ ID NO:9) and 5'-GCACGCAGGCGGTTTGTTAAG-3' (SEQ ID NO:10) (for 16S rRNA); and 5'-ACGAGGCGCTACCTAAATAGC-3' (SEQ ID NO:11) and 5'-CATGCTTAGGCGTGTGACTGC-3' (SEQ ID NO:12) (for 23S rRNA) based on the published sequences (Brosius, J., *et al.*, Proc. Natl. Acad. Sci. USA 77, 201-204 (1980); Brosius, J., *et al.*, Proc. Natl. Acad. Sci. USA 75, 4801-4805 (1978)). As a control, amplification reactions are performed without any reverse transcription process. For quantitation purposes, amplification reactions are performed under conditions in which the formation of amplified products increases exponentially using sample RNA and standard RNA preparations. The exponential amplification of products is achieved within the first 8 cycles of reaction. Therefore, all amplification reactions are performed for 7 cycles. Reaction mixtures (50 μl each) contain 91 ng of RNA, 1 x EZ *rTth* RNA

PCR buffer (Perkin Elmer), 0.3 mM of each nucleotide (dATP, dCTP, dGTP, and dTTP), 2.5 units of *rTth* DNA polymerase, 2.5 mM Mn acetate, 0.5 µl of α-^{33}P-dCTP (Easy Tides® dCTP, 2000-4000 C_i per mmol, 10 mC_i per ml, NEN Life Sciences Products, Boston, MA) and 0.15 pM of each primer. Reverse transcription reactions are carried out at 60°C for 30 min. PCR amplification reactions are carried out in triplicate for 7 cycles under the following conditions: denaturation at 94°C for 15 sec, and annealing and extension at 60°C for 30 sec. After 7 cycles, 10 µl each of the reaction mixtures are subjected to polyacrylamide gel electrophoresis (4-20% gradient gel) in a TBE buffer (Sambrook, J., *et al.*, *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y., (1989)). The resulting gels are exposed to a phosphorimager plate (Molecular Dynamics) for 2-3 hr and then scanned. The amounts of cDNA produced are quantified by measuring the intensity of each band using the manufacturer's software (Molecular Dynamics).

Generally, about 90-99% of the complexed RNA is 16S rRNA. 23S rRNA has also been found to be complexed to Era (Meier, et al., *J. Bacteriol.*, 181, 5242-5249 (1999); Meier, et al., *Microbiology*, in press (2000)). Other RNA species that have also been found to be associated with Era include 5S rRNA and Era mRNA (unpublished).

Identifying compounds with antibiotic activity using Era complexes

To identify compounds having antibacterial activity, one can search for test compounds that inhibit cell growth and/or viability by, for example, inhibiting enzymes required for cell wall biosynthesis, cell division, and DNA and protein synthesis, and/or by identifying test compounds that interact with membrane proteins. A method for identifying pharmaceutically useful antibacterial compounds comprises contacting a suitable protein or membrane preparation with a test compound, and monitoring by any suitable means an interaction, such as binding, and/or inhibition of the protein by the test compound.

The Era proteins of microorganisms are necessary for GTPase activity, which is required for bacterial cell growth. Inhibition of the Era protein, and therefore of GTPase activity, therefore represents a mechanism for bacterial inhibition.

As disclosed herein, the Era proteins of microorganisms interact with acetate ions, e.g., in the form of sodium acetate or other acetate salts, 3-phosphoglycerate, and RNA or other nucleic acids. These interactions mimic the activity of Era protein in the cell. It therefore follows that a compound that binds to or inhibits the formation of the Era-RNA complex, or that affects, preferably negatively, the interaction between the RNA or other nucleic acids and Era, acetate and Era, or 3-phosphoglycerate and Era, can be used for treating infections caused by any organism that requires Era for cell viability. Such organisms include all bacteria and Mycoplasma. These interactions can be exploited in assays aimed at identifying test compounds that inhibit one or more function(s) of Era proteins, thus leading to methods of treating bacterial or mycoplasma infections in humans or animals.

Examples of suitable means for determining the GTPase, GTP binding, or GDP binding activity of Era are disclosed in Britton, R. A., *et al.*, *J. Bacteriol.*, 179, 4575-4582 (1997); Chen, S.-M., *et al.*, *J. Biol. Chem.* 265, 2888-2895 (1990); and Zhao, G., *et al.*, *Microbiology* 145, 791-800 (1999). The present invention encompasses these and other methods appreciated by those skilled in the art.

The present invention provides methods of identifying test compounds that bind to Era proteins or Era/RNA complexes, that prevent the formation of Era/RNA complexes, that decrease the stability of Era/RNA complexes, and/or that inhibit the enzymatic activity or ligand binding activity of Era proteins or Era/RNA complexes. In addition, the present invention also provides methods of identifying compounds that affect the interaction of acetate and 3-phosphoglycerate with, and/or stimulatory effect of acetate and 3-phosphoglycerate on, Era proteins and Era/RNA complexes.

For example, in one embodiment, Era protein in the presence of RNA that complexes with the Era, or Era protein in the presence of acetate or 3-phosphoglycerate,

is used in a screen to identify a test compound that binds to and/or inhibits the activity of the Era/RNA complex or Era in the presence of the acetate or 3-phosphoglycerate. A variety of suitable screens for assaying test compounds that bind to or inhibit the complex are contemplated for this purpose. For example, the Era/RNA complex can be labeled by known techniques, such as radiolabeling or fluorescent tagging, or by labeling with biotin/avidin. Manufacturers such as Amersham and New England Nuclear supply kits facilitating such labeling. Thereafter, binding of a test compound to a labeled complex can be determined by any suitable means known in the art appropriate to the label employed. Similarly, the Era protein used in this invention can be purified and labeled, and then used in a screen to identify agents that prevent the formation of the Era/RNA complex. In addition, the enzymatic or ligand binding activity of Era protein in the presence of acetate or 3-phosphoglycerate, with and without a test compound, can be determined to ascertain the inhibitory effect of the compound on Era activity(ies).

By way of example, scintillation proximity assay (SPA; Amersham) involves labelling of a protein with biotin and labelling of a ligand that binds to the protein with an isotope (tritium is prefered in this assay). SPA beads can be coated with streptavidin, that binds to biotin on the protein. Reaction is initiated by mixing the protein, the ligand, and SPA beads together. Upon binding of the ligand to the protein, the SPA beads emit light, which can be detected using an appropriate detector. In the presence of an inhibitor, light emission is reduced, which is indicative of inhibition.

The screening methods of this invention can be adapted to automated procedures such as a PANDEX® (Baxter-Dade Diagnostics) system, allowing for efficient high-volume screening of compounds.

In a typical screen, a protein is prepared as described herein, preferably using recombinant DNA technology. A test compound is introduced into a reaction vessel containing said protein. The reaction/interaction of said protein and said compound is monitored by any suitable means. For example, binding of a test compound may be carried out by a method disclosed in U.S. Patent 5,585,277, which hereby is incorporated by reference. In this method, binding of a test compound to a protein is assessed by

monitoring the ratio of folded protein to unfolded protein, for example, by monitoring sensitivity of said protein to a protease, or amenability to binding of said protein by a specific antibody against the folded state of the protein. For example, a test compound is combined with the Era protein under conditions that cause the protein to exist in a ratio of folded to unfolded states. If the test compound binds the folded state of the protein, the relative amount of folded protein will be higher than in the case of a test compound that does not bind the protein. A similar result would be expected in a control reaction in which test compound is left out of the reaction mix.

In another method, a radioactively-labeled or chemically-labeled compound or protein is used. A specific association between the test compound and protein is monitored by any suitable means.

Protein Production Methods

Methods for producing the substantially purified Era proteins used to prepare Era/RNA complexes are described herein. Skilled artisans will recognize that proteins can be synthesized by different methods, for example, chemical methods or recombinant methods, as described in U.S. Patent 4,617,149, which is herein incorporated by reference.

The principles of solid phase chemical synthesis of polypeptides are well-known in the art, and can be found in general texts relating to this area. *See, e.g.*, H. Dugas and C. Penney, *Bioorganic Chemistry*, 54-92 (1981) Springer-Verlag, New York. Peptides can be synthesized by solid-phase methodology utilizing an Applied Biosystems 430A peptide synthesizer (Applied Biosystems, Foster City, CA) and synthesis cycles supplied by Applied Biosystems. Protected amino acids, such as t-butoxycarbonyl-protected amino acids and other reagents, are commercially available from many chemical supply houses.

The proteins used in the present invention can also be produced by recombinant DNA methods (M. P. Deutscher. 1990. Guide to protein purification. Methods in

Enzymology 182:1-894). Recombinant methods are preferred if a high yield is desired. Recombinant methods involve expressing a cloned gene, cDNA, or other nucleic acid, in a suitable host cell. The gene, etc., is introduced into the host cell by any suitable means known to persons in the art. While chromosomal integration of the cloned gene is contemplated, it is preferred that the cloned gene be maintained extra-chromosomally, as part of a vector in which the gene is in operable-linkage to a promoter.

Recombinant methods can also be used to overproduce a membrane-bound or membrane-associated protein. In some cases, membranes prepared from recombinant cells expressing such proteins provide an enriched source of the protein.

Expressing Recombinant Proteins in Procaryotic and Eucaryotic Host Cells

Procaryotes are generally used for cloning DNA sequences and for constructing vectors. For example, the *Escherichia coli* K12 strain 294 (ATCC No. 31446) is particularly useful for expression of foreign proteins. Other strains of *E. coli,* bacilli such as *Bacillus subtilis*, enterobacteriaceae such as *Salmonella typhimurium* or *Serratia marcescans*, and various *Pseudomonas* species, can also be employed as host cells in cloning and expressing the recombinant proteins of the present invention. Also contemplated is the use of various strains of *Streptococcus* and *Streptocmyces.*

For effective recombinant protein production, a structural coding sequence such as a gene, cDNA, synthetic DNA, etc. should be linked to a promoter sequence. Suitable bacterial promoters include the β-lactamase promoter (*e.g.*, vector pGX2907, ATCC 39344, contains a replicon and β-lactamase gene), lactose systems (Chang *et al., Nature* (London), 275:615 (1978); Goeddel *et al., Nature* (London), 281:544 (1979)), the alkaline phosphatase promoter, and the tryptophan (trp) promoter system (vector pATH1 (ATCC 37695)) designed for the expression of a trpE fusion protein. Hybrid promoters such as the tac promoter (isolatable from plasmid pDR540, ATCC-37282) are also suitable. Promoters for use in bacterial systems should also contain a Shine-Dalgarno sequence, operably linked to the DNA encoding the desired polypeptide. These examples are illustrative rather than limiting.

A variety of mammalian cells and yeasts are also suitable hosts. The yeast *Saccharomyces cerevisiae* is commonly used. Other yeasts, such as *Kluyveromyces lactis*, are also suitable. For expression of recombinant genes in *Saccharomyces*, the plasmid YRp7 (ATCC-40053), for example, can be used. *See, e.g.*, L. Stinchcomb, *et al.*, *Nature*, 282, 39 (1979); J. Kingsman *et al.*, *Gene*, 7, 141 (1979); S. Tschemper *et al.*, *Gene*, 10 157 (1980). Plasmid YRp7 contains the TRP1 gene, a selectable marker for a trp1 mutant.

Purification of Recombinantly-Produced Era Protein

An expression vector carrying a gene or other encoding nucleic acid of the present invention is transformed or transfected into a suitable host cell using standard methods. Cells that contain the vector are propagated under conditions suitable for expression of a recombinant protein. For example, if the gene is under the control of an inducible promoter, then suitable growth conditions would include the use of the appropriate inducer. The recombinantly-produced protein can be purified from extracts of transformed cells, from the culture medium, or both, by any suitable means.

In a preferred process for protein purification, a gene or other encoding DNA is modified at the 5' end, or at some other position, such that the encoded protein incorporates several histidine residues (viz. "histidine tag"). This "histidine tag" enables "immobilized metal ion affinity chromatography" (IMAC), a single-step protein purification method described in U.S. Patent 4,569,794, which is herein incorporated by reference. The IMAC method enables isolation of substantially pure protein starting from a crude cellular extract.

As skilled artisans will recognize, owing to the degeneracy of the code, the proteins of the invention can be encoded by a large genus of different nucleic acid sequences. In addition, the present invention also encompasses counterparts of the Era protein having the sequence shown in SEQ ID NO:2 comprising one or more

conservative amino acid changes that do not substantially affect any of the activities of the Era protein.

The ribonucleic acid (RNA) species of the present invention can be prepared using the polynucleotide synthetic methods discussed *supra,* or they can be prepared enzymatically using RNA polymerase to transcribe an appropriate DNA template.

Preferred systems for preparing the ribonucleic acids of the present invention employ the RNA polymerase from the bacteriophage T7 or the bacteriophage SP6. These RNA polymerases are highly specific, requiring the insertion of bacteriophage-specific sequences at the 5' end of a template. *See*, J. Sambrook, *et al.*, *supra*, at 18.82-18.84.

Nucleic acids complementary to the sequences disclosed herein can be prepared by the methods discussed above.

In addition to their use in preparing the Era complexes of the present invention, the probes and primers discussed herein are also useful for a variety of molecular biology techniques including, for example, hybridization screens of genomic or subgenomic libraries, or detection and quantification of mRNA species as a means to analyze gene expression. A nucleic acid fragment is provided comprising any of the sequences disclosed herein, or a complementary sequence thereof, or a fragment thereof, which is at least 15 base pairs in length, and which will hybridize selectively to *Streptococcus pneumoniae* DNA or mRNA. Preferably, the 15 or more base pair compound is DNA. A probe or primer length of at least 15 base pairs is dictated by theoretical and practical considerations. *Se, e.g.*, B. Wallace and G. Miyada, "Oligonucleotide Probes for the Screening of Recombinant DNA Libraries," In <u>*Methods in Enzymology*</u>, <u>152</u>, 432-442, Academic Press (1987).

Skilled artisans will recognize that the DNA and RNA probes or primers, or fragments thereof, can be generated by general cloning methods. (*See, e.g.*, Sambrook *et al.*, *supra*). PCR amplification, for example, using oligonucleotide primers targeted to any suitable region of SEQ ID NO:1, is preferred. Methods for PCR amplification are

widely known in the art. *See, e.g.*, PCR Protocols: A Guide to Method and Application, Ed. M. Innis *et al.*, Academic Press (1990) or U.S. Patent No. 4,889,818, which are incorporated by reference herein. A PCR comprises DNA, suitable enzymes, primers, and buffers, and is conveniently carried out in a DNA Thermal Cycler (Perkin Elmer Cetus, Norwalk, CT). A positive PCR result is determined by, for example, detecting an appropriately-sized DNA fragment following agarose gel electrophoresis.

The DNA and RNA probes or primers, or fragments thereof, can also be produced using synthetic methods known in the art. *See, e.g.*, E.L. Brown, R. Belagaje, M.J. Ryan, and H.G. Khorana, *Methods in Enzymology*, 68109-151 (1979). An apparatus such as the Applied Biosystems Model 380A or 380B DNA synthesizers (Applied Biosystems, Inc., 850 Lincoln Center Drive, Foster City, CA 94404) can be used to synthesize DNA. Synthetic methods rely upon phosphotriester chemistry (*See, e.g.*, M.J. Gait, ed., *Oligonucleotide Synthesis, A Practical Approach*, (1984)), or phosphoramidite chemistry.

The present invention also relates to recombinant DNA cloning vectors and expression vectors comprising the nucleic acids of the present invention. Preferred nucleic acid vectors are those that comprise DNA. The skilled artisan understands that selecting the most appropriate cloning vector or expression vector depends on the availability of restriction sites, the type of host cell into which the vector is to be transfected or transformed, the purpose of transfection or transformation (e.g., stable transformation as an extrachromosomal element, or integration into a host chromosome), the presence or absence of readily assayable or selectable markers (e.g., antibiotic resistance and metabolic markers of one type and another), and the number of gene copies desired in the host cell.

Suitable vectors comprise RNA viruses, DNA viruses, lytic bacteriophages, lysogenic bacteriophages, stable bacteriophages, plasmids, viroids, and the like. Preferred vectors are plasmids.

Host cells harboring the nucleic acids disclosed herein can be prepared by methods well known in the art. A preferred host is *E. coli* transfected or transformed

with a vector comprising a nucleic acid of the present invention. For example, a host cell capable of expressing a structural coding sequence as described herein can be prepared by transforming or otherwise introducing into the host cell a recombinant DNA vector comprising an isolated DNA sequence that encodes an Era protein. A preferred host cell is, for example, any strain of *E. coli* that can accommodate high level expression of an exogenously introduced coding sequence. Transformed host cells are cultured under conditions known in the art, such that the coding sequence is expressed, thereby producing the encoded protein in the recombinant host cell.

The following non-limiting examples more fully illustrate various aspects of the present invention. Those skilled in the art will recognize that the particular reagents, equipment, and procedures described are merely illustrative, and are not intended to limit the present invention in any manner.

Example 1

Production of a Vector for Expressing *S. pneumoniae* Era in a Host Cell

An expression vector suitable for expressing *S. pneumoniae* Era in a variety of procaryotic host cells, such as *E. coli*, is easily prepared. The vector contains an origin of replication (Ori), an ampicillin resistance gene (Amp) useful for selecting cells that have incorporated the vector following a tranformation procedure, and further comprises the T7 promoter and T7 terminator sequences in operable linkage to the Era coding region. Plasmid pET11A (obtained from Novogen, Madison, WI) is a suitable parent plasmid. pET11A is linearized by restriction with endonucleases NdeI and BamHI. Linearized pET11A is ligated to a DNA fragment bearing NdeI and BamHI sticky ends and comprising the coding region of the *S. pneumoniae* Era.

The Era coding region used in this construction is conveniently prepared by PCR amplification from genomic DNA using suitably designed primers. The coding region is slightly modified at the 5' end (amino terminus of encoded protein) in order to simplify purification of the encoded protein product. For this purpose, an oligonucleotide

encoding 8 histidine residues is inserted after the ATG start codon at nucleotide position 3 of SEQ ID NO:1. Placement of the histidine residues at the amino terminus of the encoded protein serves to enable the IMAC one-step protein purification procedure.

Example 2

Recombinant Expression and Purification of a Protein Encoded by the *S. pneumoniae era* Gene

An expression vector comprising the *era* gene from the *S. pneumoniae* genome as disclosed herein, which gene is operably-linked to an expression promoter, is transformed into *E. coli* BL21 (DE3)(*hsd*S *gal* λcIts857 *ind*1*Sam*7*nin*5*lac*UV5-T7gene 1) using standard procedures (see Example 4). Transformants selected for ampicillin resistance are chosen at random and tested for the presence of the vector by agarose gel electrophoresis using quick plasmid preparations. Colonies that contain the vector are grown in L broth, and the protein product encoded by the vector-borne ORF is purified by immobilized metal ion affinity chromatography (IMAC), essentially as described in US Patent 4,569,794.

Briefly, the IMAC column is prepared as follows. A metal-free chelating resin (e.g., Sepharose 6B IDA, Pharmacia) is washed in distilled water to remove preservative substances and infused with a suitable metal ion (e.g., Ni(II), Co(II), or Cu(II)) by adding a 50mM metal chloride or metal sulfate aqueous solution until about 75% of the interstitial spaces of the resin are saturated with colored metal ion. The column is then ready to receive a crude cellular extract containing the recombinant protein product.

After removing unbound proteins and other materials by washing the column with any suitable buffer, pH 7.5, the bound protein is eluted in any suitable buffer at pH 4.3, or preferably with an imidizole-containing buffer at pH 7.5.

Example 3

Protection of Era Protein from Proteolytic Digestion by Proteinase K

Era protein (100 μg/ml), proteinase K (80 μg/ml), 0.1 M Tris-HCl, pH 7.5, and test compound at a concentration in the range of from about 10^{-10} M to about 10^{-2} M are combined and incubated at 54° C for 5 minutes. Samples are removed, and undigested Era is quantified by ELISA as follows. Protease incubations are diluted 50-fold with Tris Buffered Saline (TBS). About 50 μl of diluted samples are transferred to ELISA plates and incubated for 1 hour at room temperature. The plates are washed with TBS plus 0.1% Tween-20 (TBST). About 50 μl of anti-Era rabbit serum diluted 250-fold into TBST plus 5% nonfat dry milk are added to each well and incubated 30 minutes at room temperature.

The plate wells are washed again as above, and 50 μl of goat anti-rabbit IgG alkaline phosphatase conjugate diluted 500-fold in TBST plus 5% nonfat dry milk are added to each well and incubated 30 minutes at room temperature. Wells are washed again, and 0.1 ml of 1.0 mg/ml *p*-nitrophenylphosphate in 0.1% diethanolamine is added. Color development is proportional to alkaline phosphatase antibody conjugate bound.

Example 4
Effects of Various Carbohydrates on the GTPase Activity of *E. coli* Era Proteins

The effects of various carbohydrates on the activity of *E. coli* Era proteins were determined.

Native Era protein was purified as described by Zhao, G., *et al.*, *Microbiology* 145, 791-800 (1999). GST-Era and GST proteins were affinity-purified as described by Zhao, G., *et al.*, *Microbiology* 145, 791-800 (1999). The GST-Era-RNA complex was obtained by subjecting an affinity-purified GST-Era protein preparation to gel filtration column chromatography. The void volume fractions from the column containing GST-Era associated with RNA were collected and designated as GST-Era-RNA complex, and the peak fractions with a molecular mass of 120 kDa (GST-Era dimer) were collected and designated as GST-Era (free of RNA).

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GTPase activities of the Era proteins and RNA were assayed by HPLC by measuring their ability to hydrolyze GTP to GDP in the presence or absence of 10 mM of each test compound (Zhao, G., *et al.*, *Microbiology* 145, 791-800 (1999)).

The results are shown in Table 1.

Table 1. **Effects of various carbohydrates on the GTPase activities of *E. coli* Era proteins**

Test compounds	Native Era		GST-Era		GST-Era-RNA complex	
	GTPase activity (mmol/min/mol)	Fold induction	GTPase activity (mmol/min/mol)	Fold induction	GTPase activity (mmol/min/mol)	Fold induction
Control (no compound)	17	1	4	1	61	1
Sodium acetate	50	3	39	10	84	1.3
Glyceraldehyde 3-PO_4	5	0.3	0	0	3	0.05
3-Phosphoglycerate	37	2.2	50	12.5	86	1.4
2-Phosphoglycerate	21	1.2	4	1	58	1
Glycerate	12	0.7	4	1	48	0.8
Lactate	16	1	1	0.3	62	1
Pyruvate	16	1	0	0	68	1.1

The data in Table 1 demonstrate that sodium acetate and 3-phosphoglycerate stimulate the GTPase activity of Era that is free of RNA, but did not stimulate the GTPase activity of Era that is associated with RNA. Glyceraldehyde-3-phosphate inhibited GTPase activity in all preparations. The remaining carbohydrates tested did not exhibit significant inhibitory or stimulatory activity. GST protein exhibited no GTPase activity.

Example 5

Effect of Acetate on the GTPase Activity of *E. coli* and *S. pneumoniae* Era Proteins

The effect of acetate on the GTPase activity of Era proteins was determined.

The native Era protein of *E. coli* was purified using three different columns as described by Zhao, G., *et al.*, *Microbiology* 145, 791-800 (1999). GST-ΔEra is a C-terminal deleted version of *S. pneumoniae* Era in which 67 amino acids from the C-terminal end have been eliminated. Production of this Era variant is described in Zhao, G., *et al.*, *Microbiology* 145, 791-800 (1999). The GST-ΔEra and GST-Era proteins of *S. pneumoniae* were purified first by glutathione affinity column chromatography as described by Zhao, G., *et al.*, *Microbiology* 145, 791-800 (1999), and then by Mono Q column chromatography also as described by Zhao, G., *et al.*, *Microbiology* 145, 791-800 (1999). The GST-Era-RNA complex of *S. pneumoniae* was obtained by subjecting an affinity-purified GST-Era protein preparation to gel filtration column chromatography as described by Zhao, G., *et al.*, *Microbiology* 145, 791-800 (1999). The void volume

fractions from the column containing GST-Era associated with RNA were collected and designated as GST-Era-RNA complex. All purified Era protein preparations were assayed for GTPase activity in the presence or absence of 50 mM sodium acetate. It should be noted that the particular acetate salt used in these assays is not critical.

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The results are shown in Table 2.

Table 2. **Acetate activation of the GTPase activity of Era proteins**

Enzyme	K_m (μM)		V_{max} (mmol/min/mol)	
	Acetate (50 mM)		Acetate (50 mM)	
	+	-	+	-
The native Era protein of *E. coli*	288	724	115	44
GST-ΔEra of *S. pneumoniae*	108	132	16	11
GST-Era of *S. pneumoniae*	102	491	336	24
GST-Era-RNA complex of *S. pneumoniae*	293	271	544	601

The results shown in Table 2 demonstrate that acetate did not have a significant effect on the K_m or V_{max} value of the GST-ΔEra or GST-Era/RNA complex. These results suggest that acetate stimulation of GTPase activity of Era is dependent on the presence of the C-terminal domain of the Era protein. However, acetate increased the K_m value but decreased the V_{max} value of Era that is not associated with RNA.

The invention being thus described, it is obvious that the same can be varied in many ways. Such variations are not to be regarded as a departure from the spirit and scope of the present invention, and all such modifications as would be obvious to one skilled in the art are intended to be included within the scope of the following claims.

What Is Claimed Is:

1. An Era/RNA complex, comprising:

at least one Era protein; and

at least one RNA species selected from the group consisting of 16S rRNA, 23S rRNA, 5S rRNA, mRNA of Era, an oligomeric fragment of any one of the foregoing RNA species, a probe derived from any one of the foregoing RNA species, a primer derived from any one of the foregoing RNA species, and mixtures thereof.

2. The Era/RNA complex of claim 1, wherein the length of said probe or primer is in the range of from about 6 nucleotides to about 2,900 nucleotides.

3. The Era/RNA complex of claim 1, wherein said at least one Era protein and said at least one RNA species are obtained from the same or different biological source.

4. The Era/RNA complex of claim 1, wherein said at least one Era protein and said at least one RNA species are obtainable from a microorganism selected from the group consisting of *Streptococcus sp.*, *Escherichia sp.*, *Staphylococcus sp.*, *Haemophilus sp.*, *Mycoplasma sp.*, *Mycobacteria sp.*, *Enterococcus sp.*, *Chlamydia* sp., a mutant of any of the foregoing, and progeny of any of the foregoing.

5. The Era/RNA complex of claim 4, wherein said at least one Era protein and said at least one RNA species is obtainable from a microorganism selected from the group consisting of *Streptococcus sp.*, *Escherichia sp.*, a mutant of either of the foregoing, and progeny of either of the foregoing.

6. The Era/RNA of claim 4, wherein said at least one Era protein and said at least one RNA species is obtainable from a microorganism selected from the group consisting of *Streptococcus pneumoniae, Escherichia coli, Staphylococcus aureus, Haemophilus influenzae, Mycoplasma pneumoniae, Mycobacteria tuberculosis, Enterococcus feacium, Chlamydia pneumoniae*, a mutant of any of the foregoing, and progeny of any of the foregoing.

7. The Era/RNA of claim 4, wherein said at least one Era protein is obtainable from a microorganism selected from the group consisting of *Streptococcus pneumoniae, Escherichia coli* , a mutant of either of the foregoing, and progeny of any of the foregoing.

8. The Era/RNA complex of claim 1, wherein said RNA species is16S rRNA or 23S rRNA.

9. The Era/RNA complex of claim 1, wherein 16S rRNA is the predominant RNA species present in said Era/RNA complex.

10. A method of producing said Era/RNA complex of claim 1 *in vitro*, comprising effecting *in vitro* the formation of a complex between at least one Era protein and at least one RNA species selected from the group consisting of 16S rRNA, 23S rRNA, 5S rRNA, mRNA of Era, an oligomeric fragment of any one of the foregoing RNA species, a probe derived from any one of the foregoing RNA species, a primer derived from any one of the foregoing RNA species, and mixtures thereof.

11. A method of producing said Era/RNA complex of claim 1 *in vivo*, comprising:

expressing at least one Era protein in a cell containing at least one RNA species selected from the group consisting of 16S rRNA, 23S rRNA, 5S rRNA, and mRNA of Era; and

recovering said Era/RNA complex.

12. The method of claim 11, wherein said Era protein is overexperessed.

13. The method of claim 11, wherein said Era/RNA complex is isolated chromatographically.

14. The method of claim 13, wherein said Era/RNA complex is isolated by a chromatographic method selected from the group consisting of gel filtration column chromatography, affinity chromatography, ion exchange chromatography, adsorption chromatography, hydrophobic interaction chromatography, and dye chromatography.

15. The method of claim 11, wherein said Era/RNA complex is isolated electrophoretically.

16. A method of identifying a compound that inhibits the formation of an Era/RNA complex according to claim 1, wherein said Era protein or said RNA species thereof is independently optionally labeled, comprising:

c) determining the amount of Era/RNA complex formed in the absence of said compound; and

d) comparing the amount of Era/RNA complex formed in a) with the amount of Era/RNA complex formed in the presence of said compound,

wherein any reduction in the amount of Era/RNA complex formed in b) compared to that formed in a) indicates that said compound inhibits the formation of said Era/RNA complex.

17. The method of claim 16, wherein the amount of Era/RNA complex formed in steps a) and b) is determined electrophoretically.

18. The method of claim 16, wherein at least one of said Era protein or said RNA species is labeled.

19. The method of claim 18, wherein said Era protein or said RNA species is labeled using a radiolabel, a fluorescent tag, or biotin/avidin.

20. The method of claim 16, wherein formation of said Era/RNA complex in step a) is performed by adding said compound to a mixture of said Era protein and said RNA species.

21. The method of claim 16, wherein formation of said Era/RNA complex in step a) is performed by adding said RNA species to a mixture of said Era protein and said compound.

22. The method of claim 16, wherein formation of said Era/RNA complex in step a) is performed by adding said Era protein to a mixture of said RNA species and said compound.

23. A method of identifying a compound that decreases the stability of said Era/RNA complex of claim 1, wherein said Era protein or said RNA species thereof is independently optionally labeled, comprising:

c) determining the stability of said Era/RNA complex in the absence of said compound; and

d) comparing the stability of said Era/RNA complex in a) with the stability of said Era/RNA complex formed in the presence of said compound,

wherein any reduction in the stability of said Era/RNA complex in b) compared to that in a) indicates that said compound decreases the stability of said Era/RNA complex.

24. The method of claim 23, wherein at least one of said Era protein or said RNA species is labeled.

25. The method of claim 24, wherein said Era protein or said RNA species is labeled using a radiolabel, a fluorescent tag, or biotin/avidin.

26. The method of claim 23, wherein the stability of said Era/RNA complex is determined by scintillation proximity assay or electrophoresis.

27. A method of identifying a compound that binds to said Era/RNA complex of claim 1, wherein said Era protein or said RNA species thereof is independently optionally labeled, comprising contacting said Era/RNA complex and said compound, and measuring the binding of said compound to said Era/RNA complex.

28. A method of identifying a compound that binds to said Era/RNA complex of claim 1, wherein said Era protein or said RNA species thereof is independently optionally labeled, comprising adding said compound to a mixture of said Era protein and said RNA species, and measuring the binding of said compound to Era/RNA complex formed.

29. A method of identifying a compound that binds to said Era/RNA complex of claim 1, wherein said Era protein or said RNA species thereof is independently optionally labeled, comprising adding said RNA species to a mixture of said Era protein and said compound, and measuring the binding of said compound to Era/RNA complex formed.

30. A method of identifying a compound that binds to said Era/RNA complex of claim 1, wherein said Era protein or said RNA species thereof is independently optionally labeled, comprising adding said Era protein to a

mixture of said RNA species and said compound, and measuring the binding of said compound Era/RNA complex formed.

31. A method of identifying a compound that inhibits the enzymatic or ligand binding activity of Era protein, comprising:

c) determining the enzymatic or ligand binding activity of said Era protein in the absence of said compound; and

d) comparing the enzymatic or ligand binding activity of said Era protein in a) with the enzymatic or ligand binding activity of said Era protein in the presence of said compound,

wherein any reduction in the enzymatic or ligand binding activity of said Era protein in b) compared to that in a) indicates that said compound inhibits the enzymatic or ligand binding activity of said Era protein.

32. The method of claim 31, wherein said enzymatic or ligand binding activity of said Era protein is selected from the group consisting of Era protein GTPase hydrolysis activity, Era protein GTP binding activity, and Era protein GDP binding activity.

33. The method of claim 31, wherein said determining of steps a) and b) comprises measuring Era protein GTPase hydrolysis activity.

34. The method of claim 31, wherein said determining of steps a) and b) comprises measuring Era protein GTP binding activity.

35. The method of claim 31, wherein said determining of steps a) and b) comprises measuring Era protein GDP binding activity.

36. The method of claim 31, wherein said determining of steps a) and b) comprises measuring the enzymatic or ligand binding activity of Era protein wherein said Era protein is in the form of an Era/RNA complex.

37. The method of claim 36, wherein said enzymatic or ligand binding activity of said Era protein is selected from the group consisting of Era protein GTPase hydrolysis activity, Era protein GTP binding activity, and Era protein GDP binding activity.

38. The method of claim 36, wherein said determining comprises measuring Era protein GTPase hydrolysis activity.

39. The method of claim 36, wherein said determining comprises measuring Era protein GTP binding activity.

40. The method of claim 36, wherein said determining comprises measuring Era protein GDP binding activity.

41. A method of identifying a compound that inhibits the enzymatic or ligand binding activity of Era protein, comprising:

c) determining the enzymatic or ligand binding activity of said Era protein in the absence of said compound and the presence of acetate or 3-phosphoglycerate; and

d) comparing the enzymatic or ligand binding activity of said Era protein in a) with the enzymatic or ligand binding activity of said Era protein in the presence of acetate or 3-phosphoglycerate and said compound,

wherein any reduction in the enzymatic or ligand binding activity of said Era protein in b) compared to that in a) indicates that said compound inhibits the enzymatic or ligand binding activity of said Era protein.